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Amino Acid Uptake and Metabolism by Larvae of the Marine Worm *Urechis caupo* (Echiura), a New Species in Axenic Culture

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Abstract. Axenic (bacteria-free) larval cultures of the marine echiuran worm, *Urechis caupo,* were reliably obtained by aseptically removing gametes directly from the gamete storage organs. Trochophore larvae only removed neutral amino acids from seawater as measured by high-performance liquid chromatography (HPLC). There was no detectable uptake, as measured by HPLC, of acidic or basic amino acids. Kinetic analysis showed that the transport system for alanine in 4-day-old larvae had a K_t of 4–6 μM and a J_{max} of 9–10 pmol larva^{-1} h^{-1}. Following a 50-min exposure, the majority of the radioactivity (95%) from ^{14}C-alanine was found in the trichloroacetic acid-soluble fraction. Very little label appeared as acid-insoluble material, and there was no detectable lipid biosynthesis from ^{14}C-alanine. Approximately 12% of the total alanine transported was released in the form of $^{14}CO_2$. Thin-layer chromatography of intracellular free amino acid pools demonstrated that aspartic acid and glutamic acid were radiolabeled from the alanine precursor. A comparison of the energy acquired from the transport of alanine, with the metabolic rate of 4-day-old larvae, revealed that 51% of the metabolic demand could be provided by the transport and complete catabolism of this single amino acid at a concentration of 595 nM in seawater.

Introduction

Planktotrophic (feeding) larvae of marine invertebrates must obtain food from the environment in order to supply energy for growth and metabolism (Thorson, 1946; Mileikovsky, 1971). These larvae possess anatomical adaptations to concentrate and clear particles from seawater (Strathmann, 1971; Strathmann *et al.,* 1972). However, adaptations for energy and nutrient acquisition need not exist only for the capture of particulate food. Larvae have a large surface area to volume ratio owing to their small size. Structural elaborations for locomotion and particle capture also increase the surface area (*e.g.,* molluskan velum, echinoderm ciliated bands). The total surface area of the epithelium is further enhanced by the presence of an apical brush border on certain cells (*e.g.,* Waller, 1981; Amieva and Reed, 1987). In addition to the anatomical modifications used for the capture of particles, both larval and adult soft-bodied marine invertebrates can take up dissolved organic material (DOM) directly from seawater across their body-wall (see review by Stephens, 1988).

Uptake of DOM by larvae has been primarily studied as the fluxes of free amino acids from seawater. To date, using a variety of analytical techniques, amino acid transport has been demonstrated for a number of planktotrophic larvae. Larvae of the annelids *Nereis virens* and *Neanthes arenaceodentata* accumulate radioactivity when exposed to ^{14}C-labeled amino acids in seawater (Bass *et al.,* 1969; Reish and Stephens, 1969). Amino acid influx and net flux has been reported for plutei of two species of echinoid echinoderms (*Strongylocentrotus*

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Unusual abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DOM, dissolved organic material; HPLC, high-performance liquid chromatography.

purpuratus and *Dendraster excentricus*) (Manahan *et al.*, 1983; Davis and Stephens, 1984).

Manahan (1983) examined the biochemical fate of ^{14}C from transported amino acids in two bivalve veligers, *Crassostrea gigas* and *Mytilus edulis*. The patterns of carbon assimilation were similar following a 100-min exposure to 0.5 μM ^{14}C-amino acid for *C. gigas* (glycine) and *M. edulis* (alanine). The majority of the radioactivity (75%) in the larva was localized in the cold trichloroacetic acid (TCA)-soluble fraction, 20–25% was associated with the TCA-insoluble pellet, and virtually no label was found in the lipid fraction (<2%). The production of $^{14}CO_2$ by *C. gigas* larvae, measured in parallel, represented 33% of the total glycine transport (the sum of isotope in the larva and respired radioactivity).

Based on these published accounts, most planktotrophic larvae should have the ability to remove amino acids from seawater and incorporate the acquired substrates in metabolism. However, quantitative interpretation of the rate of substrate transport and metabolism has been hampered by the fact that the majority of these experiments were conducted in the presence of bacteria. Heterotrophic bacteria can take up amino acids and other forms of DOM from seawater and use these compounds for growth and metabolism (Williams, 1975). Hence, the metabolic activity of bacteria may confound the results of studies on the ability of larvae to transport and metabolize amino acids. For instance, in adult sea urchins (*Strongylocentrotus droebachiensis*) the activity of intestinal (surface-adherent) bacteria appears to convert radiolabeled glucose to "essential" amino acids (Fong and Mann, 1980). Attempts have been made to eliminate bacteria from larval cultures by the addition of antibiotics (Millar and Scott, 1967), but the effectiveness of antibiotic treatments on marine bacteria is unpredictable and deleterious effects on larvae have been reported by D'Agostino (1972) for the crustacean *Artemia salina*. Recent advances in culturing techniques now allow the production of axenic larval suspensions without the need for antibiotics (Langdon, 1983). Aseptic collection and combination of oocytes, eggs, and sperm has provided axenic larval cultures of *Crassostrea gigas*, *S. purpuratus*, and *Dendraster excentricus* (Langdon, 1983; Manahan *et al.*, 1983; Davis and Stephens, 1984). These three species, representing two phyla, are the only ones for which studies of amino acid transport by larvae from seawater have been conducted under axenic conditions. To evaluate whether patterns of amino acid uptake are the same for all larvae, irrespective of phylogenetic affinity, or if significant differences exist between larvae from different phyla, we have developed a technique that allows the production of axenic suspensions of trochophore larvae of the echiuran worm, *Urechis caupo*.

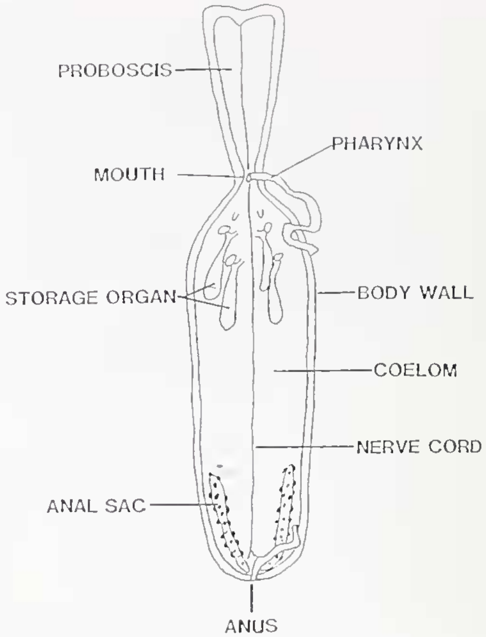

Figure 1. Ventral view of a generalized echiuran worm, drawn to display the location of the gamete storage organs (redrawn from Gould-Somero, 1975).

The echiuran, *Urechis caupo*, MacGinitie and Fisher, 1928, has a high fecundity and easily accessible gametes (Gould-Somero, 1975). The oocytes of this species have been previously used to describe biochemical changes pre- and post-fertilization (*e.g.*, Gould, 1969a, b). *U. caupo* is dioecious and gametogenesis in both sexes occurs within a spacious coelomic cavity. Mature spermatozoa and oocytes are segregated from the general cellular constituents of the coelomic fluid by three pairs of ciliated funnels, and each ciliated funnel is confluent with a gamete storage organ (Fig. 1). These storage organs can be excised allowing easy manipulation of fertilizable gametes.

We report here the development of a technique for aseptic removal of gametes and fertilization that results in axenic cultures for larvae of the echiuran worm, *Urechis caupo*. Axenic larvae were used to measure (i) rates of amino acid uptake by larvae, (ii) biochemical fates of a transported substrate, and (iii) larval metabolic rates.

Materials and Methods

Animal culture

Urechis caupo adults were obtained from Sea Life Supply Co. (Sand City, California) and maintained at 15–17°C in filtered seawater (0.2 μm, pore size, Nuclepore). Adults were removed from the tank and narcotized in 3.1% $MgCl_2$ (w/v) for 30–40 min prior to manipulation.

An incision was made through the body wall in the anterior one-quarter of a worm and continued anteriorly to the level of the proboscis, exposing the coelomic cavity. Each storage organ was clamped with a hemostat and removed by excision. All further manipulations were done, using autoclave-sterilized glassware, in a sterile transfer hood with laminar air flow (Labconco, Inc.). Each storage organ was blotted with a tissue to remove excess fluid carried over with the gamete storage organ. The storage organ was then placed in two sequential 45-s washes in 75% ethanol. Following the second ethanol wash, the storage organ was placed in sterile seawater for 45 s. Hereafter, "seawater" refers to natural seawater which was passed through a 0.2 μm (pore size, Nuclepore) filter, then autoclaved. The gametes were removed and collected by one of two methods depending on the size of the storage organs. Large storage organs were placed on tissue paper, swabbed with 75% ethanol, and the gametes removed with a flame-sterilized syringe needle. Small storage organs were opened with a flame-sterilized scalpel and the gametes collected in a beaker of seawater. Following insemination, the fertilized oocytes were placed in a 6-l Erlenmeyer flask and maintained at a temperature of 15°C. No particulate food (phytoplankton) was provided to the larvae in order to maintain axenic cultures. All experiments were conducted at 15°C.

To collect axenic trochophore larvae for experiments, the contents of a culture flask were aseptically siphoned at a slow rate onto an autoclave-sterilized 44-μm polyester mesh within a transfer hood (laminar air flow). The larvae were placed in a graduated cylinder and the larval concentration was determined by triplicate counting of known aliquots from the larval suspension.

Tests for bacteria

One ml samples of fertilized oocyte suspensions, and seawater from larval cultures, were aseptically placed in 3 ml of a sterile enriched-seawater broth (Ruby *et al.*, 1980). We assayed for contaminating bacteria by monitoring the turbidity of the seawater broth, and by further examination with 4',6-diamidino-2-phenylindole (DAPI) staining and epifluorescent microscopy (Porter and Feig, 1980). DAPI is a DNA-specific fluorescent stain that allows the detection of bacteria in the seawater broth. To test for false negative results, 1 ml of nonsterile seawater was added to the broth and assayed as described above.

Net uptake of amino acids

For experiments designed to measure the uptake of amino acids by larvae, a sufficient volume of the larval suspension was added to seawater in a sterile flask (final volume, 100 or 200 ml) to produce a larval concentration of 150–250 larvae/ml. Thirteen amino acids (made from crystalline powders, Sigma Chemical Co.) were added as a mixture to the larval suspension, producing a known amino acid concentration that ranged from 100 n*M* to 250 n*M* per individual substrate, depending on the experiment. As trochophore larvae of *Urechis caupo* swim vigorously, the experimental flask did not require continuous mixing throughout the experiment. In parallel with the uptake experiments, the amino acid mixture was added to an identical flask containing no larvae. This flask served as a control for changes in substrate concentration attributable to surface adsorption to the flask. At regular time intervals, a 500-μl sample was removed, gently passed through a 0.2-μm (pore size) polycarbonate filter held in a 13-mm filter housing (Nuclepore), and frozen. Depletion of individual amino acids from the medium was determined using high-performance liquid chromatography (HPLC). Amino acids in seawater were derivatized with *o*-phthaldialdehyde (Lindroth and Mopper, 1979) and the fluorescent derivatives separated using a sodium acetate-based buffer system (Jones *et al.*, 1981) on an Ultrasphere ODS column (4.6 cm × 7.5 mm; 3 μm particle size). The eluent profile and HPLC equipment are described elsewhere (Manahan, 1989).

The concentrations of amino acids used in our experiments were below the half-saturation constant (the K_t) of 4–6 μM for a representative neutral amino acid (see Table I). Thus, the depletion rates of individual amino acids are first-order with respect to substrate concentrations and, therefore, are nonlinear as a function of time (see Fig. 4). Any calculation of uptake rates based on end-point analyses would, under these circumstances, be invalid. At each sampling interval, the concentration for each of the 13 individual amino acids was ln-transformed to yield a linear plot. The rate of amino acid uptake by larvae was calculated from the rate of substrate depletion using the first-order depletion constant, "k", where $k = (\ln [S]_o - \ln [S]_t)/t$ (see Segel, 1976, p. 227). The depletion constant was calculated from the slope of a least-squares linear regression analysis of ln-transformed substrate concentrations with time. Values for the initial substrate concentration ($[S]_o$), and the final substrate concentration ($[S]_t$), were calculated from the regression equation based on all data points. The rate of net flux, expressed as pmol amino acid larva^{-1} h^{-1}, was calculated by multiplying the amount of each amino acid present in the mixture (*e.g.*, 20 nmol/200 ml, $[S] = 100$ n*M*) by its respective depletion constant (k), and then dividing each rate by the total number of larvae in the flask.

To determine whether the disappearance of ^{14}C-labeled alanine from the medium actually represented the net substrate flux into a larva, the change in the amount of total alanine (^{12}C and ^{14}C), as a function of time, was determined by direct chemical measurement with HPLC. The rate of influx of ^{14}C-alanine was measured by monitoring the change in the amount of ^{14}C in the medium with isotope techniques. Both the HPLC and the ^{14}C measurements were made on different aliquots of the same samples. At the start of each experiment, a known amount of ^{14}C-alanine (168 $\mu Ci/\mu mol$, New England Nuclear) was added to the same flask used to determine, by HPLC measurement, the rates of amino acid uptake into larvae. Samples of seawater were removed and filtered as described above. The depletion of total alanine in the medium (measured by HPLC) was compared to the disappearance of ^{14}C-labeled alanine (measured by liquid scintillation counting). The samples used for detection of radioactivity were acidified ($pH < 2$) for 1 day to volatilize any $^{14}CO_2$, prior to the addition of 5 ml of scintillation cocktail (Scinti-Verse, Fisher Scientific).

Kinetics of alanine transport

A known number of 4-day-old larvae was transferred into each of ten 20-ml vials (10 ml final volume). Each vial was used to measure the rate of alanine transport at a specific substrate concentration ranging from approximately 0.5 to 100 μM. To determine both the K_t and the J_{max} for the alanine transport system, the substrate concentrations that were used had to be greater than the reported concentrations of alanine in seawater (*e.g.*, Mopper and Lindroth, 1982). To accurately measure low amounts of radioactivity, relatively high concentrations of larvae (*ca.* 240/ml) were used in each experiment. To compensate for these concentrations of larvae, short time course experiments were performed. A 7–8 min experiment was conducted for each substrate concentration. At sampling intervals of approximately 1 min, a 500-μl sample was removed and layered onto 500 μl of silicone oil (Versilube F-50, General Electric Co.) in a 1.5-ml microfuge test tube. The larvae were separated from the medium by centrifugation (Beckman Model "E" microfuge, 12,500 × *g*). Following centrifugation, the supernatant and oil were removed, and the pellet of larvae collected by cutting off the bottom of the centrifuge tube. The larvae were immediately placed in a 6-ml scintillation vial and 500 μl of tissue solubilizer (Scinti-Gest, Fisher Scientific) was added. The tissue was digested for 48 h before the radioactivity was determined. Sample radioactivity was corrected, if necessary, for quenching by the addition of a ^{14}C-toluene internal standard. The slope of the regression line for each time course experiment was used to calculate the rate of alanine transport (pmol $larva^{-1}$ h^{-1}).

Comparison of transport rates for alanine, arginine, and glutamic acid

To compare the relative rates of transport, sibling larvae, from a 2-day-old xenic culture, were exposed to either an acidic, basic, or a neutral amino acid, each at a substrate concentration of 1 μM. Radiochemicals were purchased from either New England Nuclear (^{14}C-alanine, 168 $\mu Ci/\mu mol$; or I.C.N. (^{14}C-arginine, 270 $\mu Ci/\mu mol$; ^{14}C-glutamic acid, 245 $\mu Ci/\mu mol$). The procedures used to determine the transport rate for each substrate were identical to those described above for the kinetics of alanine transport.

Metabolic fate of alanine

In another series of experiments, the biochemical fate of the ^{14}C-label in the larvae was determined following the transport of ^{14}C-U-alanine (1 μCi/10 ml) from a substrate concentration of 595 nM. During the course of 1 h, two 500-μl samples containing larvae were removed from the 10-ml vial at approximately 10 min intervals. To measure the influx of radiolabeled alanine, one sample was processed and the larvae collected as described above (see "kinetics of transport"). The other sample of larvae was gently collected on a polycarbonate filter (25 mm diameter, 5.0 μm pore size, Nuclepore), and washed twice with isothermal seawater to separate the larvae from excess radioactivity in the medium. Similar filtering procedures did not cause any efflux of ^{14}C-alanine, or any rupturing and loss of ^{12}C-alanine, from larvae (see Fig. 4). Immediately after the washings, the larvae were killed by freezing on dry ice. The filters were lyophilized for at least 3 h to remove any residual water and the larval tissue was disrupted by sonication in 1 ml of distilled water (Sonics and Materials Brand, Model VC 40 fitted with a microprobe). A 50-μl aliquot of the homogenate was taken, dissolved in tissue solubilizer, and measured to determine the total radioactivity in the homogenate. A known volume of the homogenate was separated into (i) the insoluble material in cold trichloroacetic acid (TCA), (ii) TCA-soluble, and (iii) chloroform-soluble fractions, following Mann and Gallagher's (1985) modification of the procedures described in Holland and Gabbott (1971). The following steps were added to eliminate isotope carry-over during fractionation: (a) the TCA-pellet was washed three times with 100% diethyl ether to remove residual TCA and heated to dryness, (b) the chloroform extract was heated to dryness at 100°C, and (c) both TCA-insoluble and the chloroform-extractible fractions were dissolved in tissue solubilizer for 24 h prior to the addition of scintillation cocktail. The rate of production of $^{14}CO_2$ from axenic larvae was measured using the techniques of Manahan (1983). The fate of ^{14}C-alanine in the free amino acid pools of larvae was observed using two-dimensional thin-layer chromatography (Jones and Heathcote, 1966) of ethanol extracts (75%, v/v). An aliquot of the extract (2 μl) was spotted onto a 10 cm × 10 cm cellulose plate (0.1 mm thick, EM Science Brand) and the radioactivity visualized after exposure to X-ray film (XOMAT AR, Eastman-Kodak Co.).

Metabolic rates of larvae

Oxygen consumption was measured using a Clark-type polarographic oxygen electrode system, consisting of a Strathkelvin Instruments oxygen meter (Model 781), a micro-electrode (#1302), and a microrespiration chamber (RC200). The respiration chamber was calibrated to 100 μl total volume and maintained at 15°C ± 0.02°C by a water bath (Model RDL 20, Precision Instruments). Prior to experiments, the stability and self-consumption rate of the electrode were determined in isothermal seawater. After a 2-min equilibration period, following the addition of larvae, the change in oxygen tension (mm Hg) within the respiration chamber was recorded every min for at least 21 min. At the completion of each experiment ($n \geq 3$), the larvae were removed and counted (*ca.* 100 to 200 per experiment). The electrode was calibrated to zero oxygen tension by deoxygenating seawater with excess sodium thiosulfite or sodium sulfite. Calibration of the meter reading (mm Hg) with molar oxygen concentration was made by measuring the oxygen tension in a 300-ml BOD bottle filled with isothermal seawater, and then chemically determining the molar oxygen concentration using the Winkler titration method (Parsons *et al.*, 1984).

Results

Culturing the larvae of Urechis caupo *under axenic conditions*

Aseptic removal of mature gametes from *Urechis caupo*, and subsequent fertilization, yielded axenic larvae. Approximately 80% of the cultures produced in this manner tested negative for the presence of bacteria, even after 3 years of exposure to the enriched seawater broth. For axenic cultures, DAPI-stained gametes, larvae, and culture water all tested negative for bacteria. All broths inoculated either with (i) nonsterile seawater or (ii) xenic suspensions of gametes or larvae, were visibly cloudy after 24–48 h, eliminating the possibility of false-negative results for the axenic cultures. Examination of sibling axenic and xenic larvae by light microscopy revealed no distinguishable difference in larval morphology.

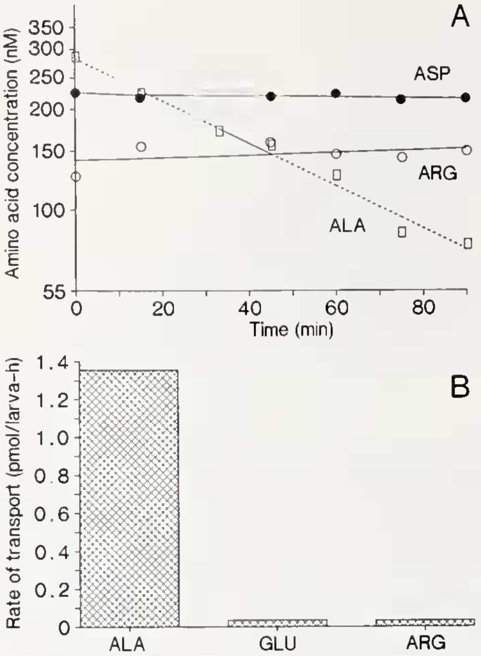

Figure 2. (A) Net flux of representative acidic, basic and neutral amino acids by 2-day-old *Urechis caupo* trochophore larvae (265 ml^{-1}), as measured by high-performance liquid chromatography (HPLC). The slopes of the regression lines for the net flux rates of aspartic acid (solid circles) and arginine (open circles) were not statistically significant from zero; the uptake of alanine (rectangles) was statistically significant ($P \leq 0.001$). (B) The rate of transport by sibling larvae of ^{14}C-alanine ($r^2 = 0.99$), ^{14}C-glutamic acid ($r^2 = 0.89$), and ^{14}C-arginine ($r^2 = 0.89$), each measured separately at a substrate concentration of 1 μM (258 larvae/ml).

Net uptake from amino acid mixtures

Trochophore larvae of *Urechis caupo* took up only neutral (polar and nonpolar) amino acids from seawater. There was no statistically significant influx of either acidic or basic amino acids from the experimental mixture of 13 individual amino acids (Fig. 2A). This result was observed for three other cultures of axenic larvae, obtained from independent spawnings. The possibility that competition between the substrates caused this observation was eliminated by comparing the rates of transport for alanine, arginine, and glutamic acid in the absence of other amino acids (Fig. 2B). From a substrate concentration of 1 μM, where the transport rate of each of the three substrates was measured separately, alanine was transported at a rate of 1.35 pmol larva^{-1} h^{-1}; glutamic acid at a rate of 0.03 pmol larva^{-1} h^{-1}; and arginine was transported also at a rate of 0.03 pmol larva^{-1} h^{-1}. Thus, the rates of transport of Arg and Glu were only 2.1% of that for alanine at an identical concentration. Figure 3 shows the rates of uptake for each of 13 individual amino acids. The total rate of uptake for the 9 neutral

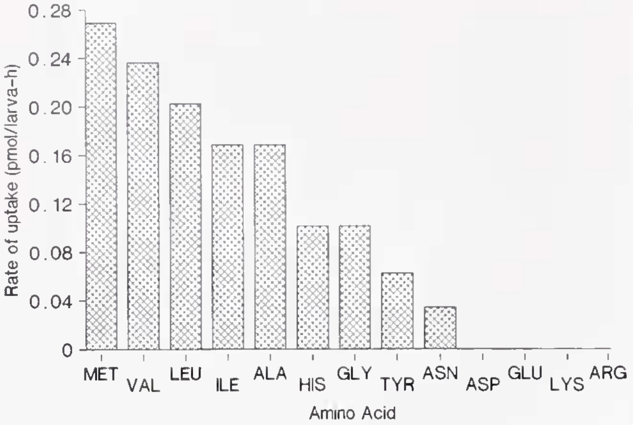

Figure 3. Depletion of 13 amino acids from the medium in the presence of trochophore larvae of *Urechis caupo* (250 larvae ml^{-1}) determined by high-performance liquid chromatography. The rate of amino acid uptake was calculated from the first order depletion constant (k) (see text). The concentration of each amino acid at the start of the experiment was 100 n*M*; where no histogram bars are presented the change in the ln-transformed substrate concentrations were not statistically significant from a slope of zero. The r^2 values for all statistically significant rates were >0.90.

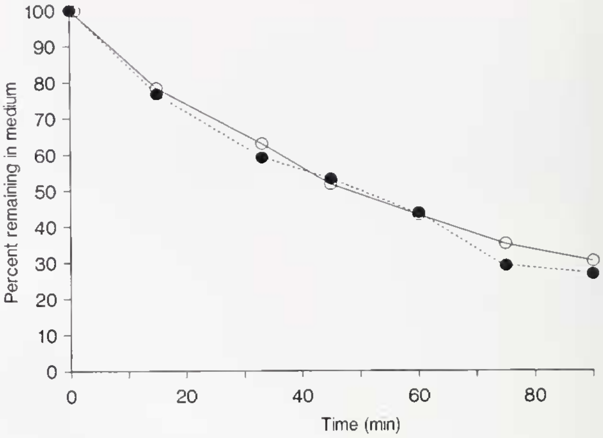

Figure 4. A comparison of influx and net flux of alanine from the medium in the presence of 1-day-old axenic *Urechis caupo* larvae (297 larvae ml^{-1}). Net flux was determined by HPLC (solid circles), and influx was measured by isotope techniques (open circles). The concentration of alanine at the beginning of the experiment was 207 n*M*.

amino acids (His is neutral at the pH of seawater) was 1.34 pmol amino acid larva^{-1} h^{-1}. In the absence of larvae, there was no detectable change in the concentration of any amino acid in the flask.

The influx of alanine equaled the net substrate flux as shown in Figure 4. The rate of influx, measured as the depletion of ^{14}C-alanine from the medium, was 0.23 pmol larva^{-1} h^{-1} ($r^2 = 0.99$); the net flux of alanine, measured by HPLC, was 0.24 pmol larva^{-1} h^{-1} ($r^2 = 0.99$). The starting substrate concentration was 207 n*M*. A comparison of the regressions for both these rates showed that there was no statistically significant difference between these rates (VR = 0.14, $F_{.05[1,10]} = 4.96$).

Kinetics of alanine transport

A graph showing the influence of substrate concentration on the rate of transport produced a rectangular hyperbola (Fig. 5). Linear transformations (Eadie-Hofstee) were characteristic of Michaelis-Menten kinetics (Fig. 5, see inset). The kinetics of alanine transport by 4-day-old trochophores were examined for both axenic and xenic larvae (nonsibling cultures). Axenic larvae had a K_t of 4 μM for alanine and a J_{max} of 9–10 pmol larva^{-1} h^{-1} (Table I). Nonaxenic larvae had very similar kinetics, with a K_t of 5–6 μM and a J_{max} of 9–10 pmol larva^{-1} h^{-1} (Table I).

Metabolic fate of alanine in larvae

From a concentration of 595 n*M*, the influx of ^{14}C-U-alanine into 4-day-old larvae was 1.72 pmol larva^{-1} h^{-1} (Table II). A second culture of 7-day-old larvae transported ^{14}C-U-alanine (595 n*M*) at a rate 1.97 pmol larva^{-1} h^{-1} (Table II). For both cultures, the majority (>95%) of the radioactivity was recovered in the TCA-soluble pool and there was no detectable radioactivity in the lipid fraction. Simultaneous measurement of $^{14}CO_2$ production, expressed as alanine equivalents, showed that 0.25 pmol and 1.10 pmol larva^{-1} h^{-1} were released by 4- and 7-day-old larvae, respectively.

Autoradiographic analysis of TLC-separated amino

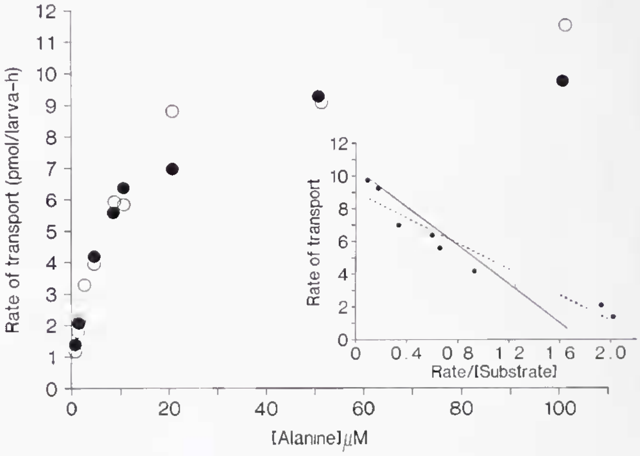

Figure 5. Effect of substrate concentration on the rate of alanine transport by axenic (solid circles) and xenic (open circles) 4-day-old *Urechis caupo* larvae. The concentration of larvae was 119 ml^{-1} (axenic experiments, n = 8) and 127 larvae ml^{-1} (xenic experiments, n = 10). Each data point (= n) represents the slope of a single time course experiment (all r^2 values > 0.95). The inset shows Eadie-Hofstee plots of the data.

Table I

A comparison of kinetic constants for alanine transport by axenic and xenic Urechis caupo *larvae (4-day-old)*

Larval condition Axenic larvae	Linear transformation Eadie-Hofstee	 Lineweaver-Burke
K_t (μM)	3.9	4.3
J_{max} (pmol larva^{-1} h^{-1})	9.0	10.0
r^2 (n = 8)	0.92	0.97
Nonaxenic larvae		
K_t (μM)	5.9	5.0
J_{max} (pmol larva^{-1} h^{-1})	10.5	9.1
r^2 (n = 10)	0.93	0.99

ANOVA	SS	df	MS	VR	F_{05}
Between slopes (K_t)	6.50	1	6.5	6.50**	4.60
Between constants (J_{max})	0.27	1	0.27	0.27ns	
Residual	14.40	14			
Total	178.17	17			

**Significant at the 99% level.

acid pools of larvae showed that most of the alanine remained intact after a 1-h exposure. However, carbon from ^{14}C-alanine also appeared in both glutamic acid and aspartic acid.

The metabolic rate of Urechis caupo *larvae*

The metabolic rate of axenic 4-day-old larvae was 10.1 ± 0.41 pmol O_2 larva^{-1} h^{-1} (mean ± 1 SE; n = 3). These rates of oxygen consumption were determined for larvae which were from the same culture as those that were used to determine rates of ^{14}C-alanine influx and metabolism (see Table II).

Discussion

A reliable method has been developed to produce axenic larvae of the marine echiuran worm *Urechis caupo.* Cultures of axenic larvae have been used to determine (i) the uptake rates of amino acids from seawater, (ii) the fate of a specific substrate following transport, and (iii) the metabolic rate. This has allowed a direct comparison, in the absence of competing species, of the contribution from a specific fraction of the DOM pool to the metabolism of *U. caupo* larvae.

Axenic *Urechis caupo* larvae only took up neutral amino acids from seawater, as measured by HPLC (Figs.

Table II

Metabolic fate of the ^{14}C-label following transport of ^{14}C-U-alanine by axenic Urechis caupo *larvae*

	Uptake	Metabolic components CO_2	 % TCA-insoluble[a]	 % TCA-soluble[b]	 % Lipid[c]
4-day-old:					
Rate (pmol Ala larva^{-1} h^{-1})	1.72	0.25	5	95	0
r^2 (n)[d]	0.99 (6)	0.96 (5)			
7-day-old:					
Rate (pmol ala larva^{-1} h^{-1})	1.97	1.10	3	97	0
r^2 (n)[d]	0.99 (5)	0.96 (6)			

[a] Percent of total radioactivity appearing in the cold TCA-insoluble fraction (macromolecules).

[b] Percent of the total radioactivity appearing in the cold TCA-soluble fraction (small molecular weight compounds).

[c] Percent of the total radioactivity in the larva contained in the chloroform-extractible fraction (lipid).

[d] "n" equals lthe number of samples used to determine the presented rate.

2A, 3). Although significant removal from the medium of either acidic or basic amino acids could not be measured, larvae do have a small transport capacity for glutamic acid and arginine, at only 2% that of the alanine transport rate (Fig. 2B). This difference in the transport capacity for acidic, basic, and neutral amino acids has also been reported for pluteus larvae of the sea urchin *Strongylocentrotus purpuratus* (Manahan *et al.*, 1983) and the sand dollar *Dendraster excentricus* (Davis and Stephens, 1984). The rate of disappearance of ^{14}C-alanine (influx) from the medium, in the presence of axenic larvae, is an accurate measurement of net flux (Fig. 4). The appearance of alanine in the tissue, therefore, represents a net gain to the larva (*cf.* Johannes *et al.*, 1969).

Kinetic analysis of the rate of alanine transport into *Urechis caupo* trochophores indicated that the transport system is adapted to function with maximal efficiency at relatively low substrate concentrations ($K_t = 4–6\ \mu M$, Table I). The y-intercepts (J_{max}) for the kinetic data, calculated from the regression of the transformed data (Eadie-Hofstee), were not statistically different between 4-day-old axenic and xenic larvae (see ANOVA, Table I). However, the values for K_t were statistically different ($VR = 6.50$, $F_{.05[1,14]} = 4.60$). The fact that there was no difference in the maximal rate of alanine transport, and the similarity of the K_t values, suggest that bacteria did not have a significant influence on alanine transport by xenic larvae (Fig. 5, Table I). The kinetic constants for alanine transport by *Urechis caupo* larvae compare favorably with literature values for amino acid transport by other embryos and larvae. Epel (1972) described the kinetics of alanine, glycine, and valine transport in fertilized eggs of the sea urchin *Strongylocentrotus purpuratus*. The kinetics of all three substrates are characterized by both a low K_t (1–2 μM) and a low J_{max} (1.4–2.5 pmol substrate larva^{-1} h^{-1}). Manahan (1983) reported that the veliger larvae of *Crassostrea gigas* had a K_t of 3.7 μM and a J_{max} (25.7 pmol larva^{-1} h^{-1}) for glycine transport. He also measured a similarly low K_t (3.5 μM) and a lower J_{max} (10.1 pmol larva^{-1} h^{-1}) for alanine transport by *Mytilus edulis* veliger larvae. Davis *et al.* (1985) examined the kinetics of serine and leucine transport by 1-day-old *S. purpuratus* embryos and found low values for K_t of 1.3 μM (leucine) and 3.6 μM (serine). The maximal rate of substrate transport was 2.5 and 6.9 pmol substrate larva^{-1} h^{-1}, respectively, for the two amino acids.

Following exposure to ^{14}C-alanine, *Urechis caupo* larvae use this substrate in anabolic and catabolic pathways. The majority (95%) of the intracellular radioactivity was associated with the TCA-soluble pool (Table II). This analytical fraction contains small molecular weight compounds and, thus, may represent an intracellular reservoir of compounds that will be subsequently used in metabolic reactions. In the current experiments, it was not surprising that the amount of radioactivity in the acid-insoluble fraction was low (<5%). Under the experimental conditions used in the present study, the larvae were not fed, and thus a high rate of net protein synthesis was unlikely. However, in veliger larvae of the bivalve *Crassostrea gigas*, which had been fed phytoplankton, Manahan (1983) reported that 20–25% of the radioactivity was localized in acid-insoluble material. The ethanol-extractable amino acids in *U. caupo* trochophores were separated by TLC and those containing the ^{14}C-label were detected with autoradiography. Following a 1-h exposure, the ^{14}C-label from ^{14}C-alanine appeared in alanine, aspartic acid, and glutamic acid. Four-day-old trochophore larvae released 12% of the total transported ^{14}C-amino acid carbon as $^{14}CO_2$. Although the uniform labeling of the isotope precludes any estimate of the precise energy derived from alanine breakdown, it is clear that this substrate is used in catabolic reactions.

If the amount of energy gained from the transport of alanine is compared to the total energy requirements of a larva (oxygen consumption), an indirect measure of the energetic significance of the transported substrates can be made. This comparison makes no assumptions about metabolic coupling between these two processes, but merely provides a means to evaluate the potential input from this mode of nutrient acquisition (Stephens, 1963). At an alanine concentration of 595 nM, trochophore larvae (4-day-old) had a total uptake rate of 1.72 pmol alanine larva^{-1} h^{-1} (Table II). Larvae of the same age, from the same culture, had a metabolic rate of 10.1 pmol O_2 larva^{-1} h^{-1}. The complete combustion of 1.72 pmol of alanine would require 5.16 pmol O_2 (1 mol alanine requires 3 mol O_2). Thus, a transport rate of 1.72 pmol alanine could account for a metabolic rate of 5.16 pmol O_2 larva^{-1} h^{-1}, or 51% of the measured metabolic rate of these larvae (10.1 pmol O_2 larva^{-1} h^{-1}). The measured rate of alanine transport at 595 nM was 1.72 pmol larva^{-1} h^{-1}, which was higher than that calculated from kinetic constants (given in Table I), for axenic larvae at the same substrate concentration (rate = 1.19 pmol larva^{-1} h^{-1}). Nonetheless, for this comparison we feel justified in using the measured value of 1.72 pmol larva^{-1} h^{-1} because both the metabolic rate and the transport rate were determined, on the same day, for the same culture of axenic larvae.

Dissolved organic carbon (DOC) in seawater ranges from 500 to 5800 μg C l^{-1}, and represents nearly ten times the amount of carbon in particulate form (Williams, 1975; Sugimura and Suzuki, 1988). Of the total DOC, free amino acids represent less than 1%, and ambient concentrations in surface seawaters range from less than 10 nM to 500 nM (Christensen and Blackburn, 1980; Mopper and Lindroth, 1982; Carlucci *et al.*, 1984; Fuhrman and Bell, 1985). Near, and within, marine sediments, the values for amino acid concentrations are 1–2 orders of magnitude higher (Clark *et al.*, 1972; Henrichs and Farrington, 1979). Although the concentrations of total free amino acids in seawater may be relatively low, it is clear that *Urechis caupo* larvae have the ability to transport these molecules at rates sufficient to provide a caloric equivalent to 51% of the metabolic rate. Of course, the rate of amino acid transport that is realized in nature will depend on the ambient substrate concentrations in the immediate environment of the larvae. The adults of *U. caupo* are infaunal inhabitants of estuaries and embayments, and their larvae will be exposed to the higher substrate concentrations of DOM found in these habitats. The demonstrated ability of *U. caupo* larvae to acquire amino acids from seawater, to metabolize them, and potentially gain a significant amount of energy, suggests that the capacity to exploit this resource is important. During periods of low particulate food availability, the ability of a larva to use another energy and nutrient source may allow a normal developmental rate to be maintained. Alternatively, if particulate food is not limiting, the additional energy from DOM could be used to accumulate energy reserves.

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